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Sub-minimum Inhibitory Concentration of Streptomycin and Cephaloridine- Induced Capsular Polysaccharide Production in *Escherichia coli K-12* Increases Biofilm Formation in a Wzy-transporter Dependent Manner

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Biofilms are bacterial communities that adhere and endure on solid surfaces. They are known to contribute to antibiotic resistance and disease. In our study, we focused on the relationship between capsule and biofilm formation. *cpsB* is a gene that encodes an enzyme required for the synthesis of colanic acid, a major component of capsular polysaccharides. The Wzy-system, a membrane transporter encoded by the genes *wza-wzb-wzc*, is essential for surface expression of capsule. We investigated the role of the *cpsB* gene and Wzy-transporter genes in capsule production and biofilm formation. Sub-minimum inhibitory concentrations of streptomycin and cephaloridine were used to induce capsule formation in *Escherichia coli* K-12 wildtype, mutants Δ*wza*, Δ*cpsB*, and the strain CWG655, lacking the gene cluster *wza-wzb-wzc*. Biofilm biomass was assessed in the presence of different levels of capsule induced by sub-minimum inhibitory concentration antibiotic treatment. We observed that sub-minimum inhibitory concentration-induced capsule production is important for biofilm formation. Increased capsule production in *Escherichia coli* K-12 wildtype induced by sub-minimum inhibitory concentration of streptomycin and cephaloridine correlated with an increase in the amount of biofilm detected. Additionally, we show that the mass of the biofilm is related to genes encoding protein related to the Wzy-transport system.

Bacterial biofilm formation and development is of substantial concern in hospitals and care homes since the discovery of their significance in human pathogenesis (1). Biofilms are described as bacterial communities that attach and endure on solid surfaces (2). They are surrounded by a matrix of hydrated extracellular polysaccharides along with water-channels for nutrient movement and retention (3). *Escherichia coli* K-12 (*E. coli* K-12) is one of the many bacterial strains that can form biofilms (3).

Different components are required for each step in the development of biofilm, such as the extracellular polysaccharide colanic acid, which is critical in establishing complex structures during the maturation of the biofilm (4). An essential enzyme in the colanic acid biosynthesis pathway is mannose-1-phosphate guanylyltransferase, encoded by the *cpsB* gene (5). In addition to its role in biofilm formation, colanic acid is also known to be a key component of the *E. coli* capsule (3). Capsules are a bacterial defense mechanism involved in environmental interactions; they are primarily formed by capsular and extracellular polysaccharides (6).

In *E. coli* K-12, capsule surface expression is dependent on the Wzy pathway which functions to transport different components of the capsule, including colanic acid from the E. *coli* cytoplasm to the cell surface (7). Within this pathway, a specific oligomeric lipoprotein, Wza, is present in the outer membrane (8). Wza provides a channel for the transport and surface expression of capsular polysaccharides outside of the cell (8). Studies using *wza* null mutants demonstrated that treatment with sub-minimum inhibitory concentrations (sub-MICs) of streptomycin led to lower levels of CPS in the mutant compared to the wildtype (WT), indicating *wza* is important for transportation of CPS outside of the cell (9). CWG655, a strain with a complete knockout of the *wza-wzb-wzc* genes, produced no capsule at all (8).

Ganal *et al.* previously demonstrated that exposure to sub-MIC of the aminoglycoside, streptomycin, led to increased levels of CPS in *E. coli* (10). In a separate study, Hoffman *et al.* established that treatment with streptomycin resulted in the induction of biofilm formation in clinical isolates of *E. coli* (11). Similar effects have also been seen on *E. coli* CPS induction upon usage of sub-MIC of cephaloridine, a beta-lactam antibiotic (12). This rapid, reversible, and non-mutational induction of capsule synthesis has been shown to be important for pathogenicity in response to antibiotic stress in *Acinetobacter baumannii* (13).

As of current knowledge, no correlation between antibiotic-induced capsule formation and increased biofilm production exists. We explored the connection between these two factors using biofilm-forming *E. coli* K-12 WT and null mutants that are unable to produce essential proteins involved in CPS production or surface expression. We hypothesized that sub-MIC of antibiotic-induced capsule formation will increase biofilm formation. To test our hypothesis, sub-MIC of streptomycin and cephaloridine were used to induce capsule formation in *E.coli* K-12 WT and various mutants. Furthermore, the amount of biofilm biomass was evaluated in the presence of different levels of capsule induced by sub-MIC of antibiotics. We observed a positive correlation between the capsule production induced by sub-MIC of antibiotics and the amount of biofilm biomass. The WT displayed greater CPS and biofilm levels under sub-MIC antibiotic conditions, compared to the untreated samples. Also, the Wzy-transporter was more important in formation of biofilm than the *cpsB* gene.

MATERIALS AND METHODS

Bacterial strains, media preparation, and growth conditions. *E. coli* K-12 BW25113 (genotype: F-, Δ(*araD- araB*)567, Δ*lacZ4787*(::rrnB-3), LAM-, rph-1, Δ(*rhaD-rhaB*)568, hsdR514), *E. coli* K-12 JW2047-1 (F-, Δ(*araD- araB*)567, Δ*lacZ4787*(::rrnB-3), LAM-, Δ*wza-760::kan*, rph-1, Δ(rhaD- rhaB)568, hsdR514) and *E. coli* JW2034 (F-, Δ(*araD-araB*)567, Δ*lacZ4787*(::rrnB-3), λ-, Δ*cpsB747::kan*, rph-1, Δ(*rhaD-rhaB*)568, hsdR514) were obtained from the MICB 421 culture collection (Department of Microbiology and Immunology, University of British Columbia). *E. coli* CWG655 [$wza_{22\ min}$::*aadA* Δ(*wza-wzb-wzc*)$_{K30}$::*aphA3*] was provided for use in the MICB 421 course by Dr. Chris Whitfield at the University of Guelph. BW25113 is denoted as wildtype (WT), JW2047-1 is referred to as the Δ*wza* mutant, the JW2034 is referred to as the Δ*cpsB* mutant and the CWG655 is denoted as CWG655. Overnight cultures of the WT, Δ*wza*, Δ*cpsB* and CWG655 strains were prepared by inoculating 5ml of Luria-Bertani (LB) medium (1% Tryptone, 0.5% Yeast Extract, 0.5% NaCl) in 18 x 150mm glass test tubes with a loopful of the respective strains. The inoculated cultures were grown overnight on a shaker at 200rpm at 21°C. Cultures that were incubated overnight were diluted to a concentration of approximately $2.5x10^8$ cells/ml (OD_{600} = 0.5) using a Beckman DU Series 500 Spectrophotometer the next day for use in subsequent protocols.

The minimum inhibitory concentration (MIC) assay. Overnight cultures were standardized to $0.5OD_{600}$ and a 40 times dilution was performed to obtain a concentration of $6.25x10^6$ cells/ml. A two-fold serial dilution was performed in a Falcon 353072 96-well plate to obtain wells containing 0 µg/ml, 1 µg/ml, 2 µg/ml, 4 µg/ml, 8 µg/ml, 16 µg/ml, 32 µg/ml, and 64 µg/ml of streptomycin (Sigma - S6501-50G) or cephaloridine (Sigma - C3519) antibiotics in LB. Each antibiotic concentration was tested in duplicate and 10µl of each culture was added to each well. The 96-well plate was then incubated overnight at 21°C. The well with the lowest antibiotic concentration that did not demonstrate growth corresponded with the MIC. This was determined by visual observations and by reading the plate on BioTek Micro-volume Plate Reader.

Subinhibitory antibiotic pretreatment. 6µl of diluted overnight culture ($0.5OD_{600}$) for each of the strains was added into tubes of 5ml LB media containing either LB only or half of the previously determined MIC of cephaloridine or streptomycin. All twelve cultures were incubated overnight for approximately 12 hours at 21°C under shaking conditions in order to induce capsular polysaccharide formation.

Surface polysaccharide extraction and quantification. Surface polysaccharides were quantified using a modified phenol-sulfuric acid method (14). After sub-minimum inhibitory antibiotic pretreatment, 1ml of each diluted *E. coli* overnight culture (OD_{650} = 0.5) was centrifuged at 14,600 x g for 5 minutes using an Eppendorf 5415D Microcentrifuge. The same centrifugation conditions were used for all the following steps. The cells were then washed by resuspending in 1ml of 50mM NaCl. The centrifugation and wash was repeated four times. Cells were pelleted and resuspended in 1ml of 50mM EDTA. They were then incubated at 37°C on the shaking rack for 60 minutes to dissociate LPS and release the outer membrane from cells. Cells were again pelleted and the resultant supernatant, containing LPS and any tightly associated capsule, was subjected to a phenol-sulfuric acid colourimetric carbohydrate quantification. The steps were as follows: carbohydrate standards were prepared by diluting a carbohydrate stock solution (1:1 mixture of sucrose and fructose dissolved in distilled water with a final concentration of 1mg/ml of carbohydrate) into 1ml aliquots for final concentrations of 0, 30, 60, 90, and 120µg/ml. 400µl of each sample, carbohydrate standards, and a negative control of 50mM EDTA were each mixed with 400µl of 5% phenol in glass test tubes. 2ml of 95% sulfuric acid were added to each tube and, colour was allowed to develop for 10 minutes at room temperature with gentle mixing. Colour intensity was then measured at 490nm using a Spectronic 20^+ spectrophotometer and was normalized to the carbohydrate standards to calculate carbohydrate concentration.

Capsule staining. The initial procedure for capsule isolation was adapted from Brimacombe *et al*. (14). After sub-minimum inhibitory antibiotic pretreatment, 1ml of each *E. coli* sample was harvested by centrifugation, and resuspended in 1ml of 1% skim milk powder (w/v). A loopful of each re-suspended culture was spread onto a clean microscope slide and air-dried. The smears were stained for 5 minutes using 1% crystal violet, copper sulphate, which served a dual function as both a decolourizer and counterstain to replace the crystal violet in the capsule only. Slides were air-dried and subsequently examined using an oil-immersion phase contrast microscopy at 100X magnification. A second capsule stain was performed using a modified Gin's capsule stain (15). Using aseptic technique, mixed one loopful of each of India ink, water, and bacterial colony of interest onto a clean glass slide. The mixed droplet was then spread flat across the slide using the border of another slide and air dried. The slide was then flooded with 1% crystal violet for 1 minute, washed gently with water, and left to air dry. Slides were examined using oil-immersion light microscopy at 100X magnification.

Biofilm growth and assay. The procedure for biofilm biomass quantification was adapted from O'Toole and Ren *et al*. (16, 17). A 40 times dilution was performed on each of the diluted overnight cultures to obtain a concentration of $6.25x10^6$ cells/ml. 5µl of each strain was added into the wells of a Falcon 353072 96-well plate containing 100µl of LB media or LB media containing the previously determined sub-MIC of streptomycin or cephaloridine at 0.5µg/ml. Seven replicates were done for each sample with one row serving as a control (no culture was added). The plate was then incubated at 21°C without shaking. The OD reading at 600nm was taken to quantify the total growth of the cells after 24, 48, 72 and 96 hours of incubation. The amount of biofilm was measured after 96 hours of incubation. The plates were washed three times with water to remove unattached cells and media components. The biofilms in each well were stained for 15 minutes at room temperature using 125µl of 0.1% crystal violet. The extra dye was removed using three washes with water and the plates were kept upside down to dry for 3 hours (or overnight). To quantify the biofilm, 125µl of 30% acetic acid in water was added to each well and incubated for 15 minutes at room temperature to solubilize the crystal violet dye. 10µl of the solubilized crystal violet solution was diluted 10 times into 90µl of 30% acetic acid in a new flat-bottomed 96-well plate and absorbance was measured at 550nm. The 550nm reading of the LB control was subtracted from all samples when plotting.

RESULTS

The sub-MIC concentrations determined by the MIC assay for *E. coli* WT, mutants Δ*wza,* Δ*cpsB,* and the CWG655 strain are 0.5 µg/ml. The MIC of streptomycin and cephaloridine was comparable among WT and different mutants (Table 1). However, we observed an 8 or 12-fold increase for the CWG655 mutant in streptomycin compared to the other results. In this experiment, the sub-MIC was defined as a concentration of antibiotic below the experimentally found lethal dose. Following the MIC assay, the sub-MIC was determined to be 0.5µg/ml for both

streptomycin and cephaloridine, and was used on the WT and each mutant strain in order to grow all cells in consistent conditions. This sub-MIC condition was used to induce capsule and biofilm formation.

TABLE 1 Minimum inhibitory concentrations of streptomycin and cephaloridine for treatment of *E. coli* K-12 WT and mutant strains incubated at 21°C for 24 hours.

	MIC (μg/ml)	
Strain	Streptomycin	Cephaloridine
WT	2	2
wza strain	2	2
cpsB strain	4	1
CWG655 strain	32	1

***E. coli* WT capsule formation is increased following sub-MIC streptomycin and cephaloridine pretreatment.** The total amount of carbohydrate quantified using the phenol-sulfuric acid assay correlates to the amount of capsular polysaccharide produced by each strain (14). We expected to see higher amounts of carbohydrate in the WT strain after pretreatment with sub-MIC antibiotics, since this was observed in literature (10, 12). As shown in Figure 1, pretreatment with sub-MIC streptomycin for the *E. coli* K-12 WT sample resulted in a 3-fold increase in the amount of quantified carbohydrate, while cephaloridine resulted in a 3.3-fold increase in carbohydrate, compared to the control *E. coli* K-12 WT grown in LB media. Cephaloridine also showed to induce greater amounts of CPS than streptomycin for all strains. It was also found that all the antibiotic pretreated capsule mutant strains had lower amounts of total carbohydrates compared to similarly pretreated *E. coli* K-12 WT samples.

The two capsule staining methods yield no observable differences in capsule size between mutants and WT. A modified version of Anthony's capsule stain was performed to visually confirm capsule quantification results. Visual observations were inconclusive as the slide backgrounds did not provide enough contrast to confidently determine the presence of capsule, which would appear as a white halo surrounding purple cells (see Figure S1 in the supplemental material). A second stain, known as a modified Gin's capsule stain was performed and involved the use of black India ink which would theoretically provide a clearer sample image to determine the capsule presence and size (Figure S2). As a result, bacterial cells stained by crystal violet showed up purple, surrounded by a thin white halo, representing the unstained capsule that was clearly distinguished from the grainy black background (Figure S2). These white halos were seen across all strains of cells treated at all conditions of growth. However, there was no microscopically observable difference in capsule size across the WT and mutant strains (Figure S2).

Growth is observed in the 96-well plate while under sub-MIC treatment. Total cell growth was measured every 24 hours to demonstrate that the *E. coli* mutants, Δ*wza*, Δ*cpsB*, and the CWG655 strain, did not have an intrinsic deficiency in cell division compared to wildtype. Also, sub-MIC of cephaloridine and streptomycin at 0.5 μg/ml did not impaired the growth of *E. coli* K-12 WT and different mutants. Growth, as indicated by a 5-fold increase in optical density compared to initial readings, was observed in all strains under all conditions at 24 hours of incubation (Figure 2). Continued growth was observed beyond 24 hours of incubation for all strains (Figure 2). Greater growth was achieved in LB media than with sub-MIC antibiotic treatment for Δ*cpsB* and CWG655 but not for Δ*wza* and WT. Greater growth overall at 96 hours was achieved in WT and CWG655.

Sub-MIC treatment of cephaloridine or streptomycin appears to increase biofilm formation in WT and Δ*cpsB* mutant. Streptomycin and cephaloridine induced 1.6-fold and 1.4-fold increase in the amount of biofilm formed by *E.coli* WT respectively, compared to untreated WT cells (Figure 3). A similar trend of antibiotic-induced elevation in biofilm formation could be observed in the Δ*cpsB* mutant. The CWG655 strain was completely deficient in biofilm formation in both LB media and LB media containing sub-MIC level of streptomycin and cephaloridine. Δ*wza* had partial functionality to produce biofilm compared to WT and Δ*cpsB* mutant. The Δ*wza* strain showed 5.2-fold and 3.0-fold decrease in the amount of biofilm produced compared to WT and the Δ*cpsB* mutant respectively in LB media (Figure 3).

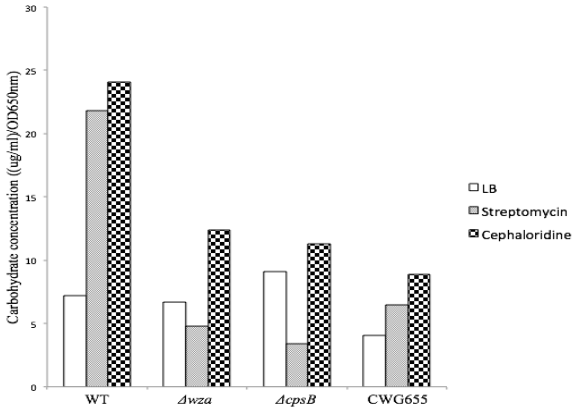

FIG 1. Effect of sub-MIC pretreatment of streptomycin and cephaloridine at 0.5 μg/ml on the total carbohydrate levels in *E. coli* K-12 WT, Δ*wza*, Δ*cpsB*, and CWG655 mutants. The strains were grown for 24 hours at 21°C, and total carbohydrate concentrations were measured using a phenol-sulfuric acid assay. Data was obtained from one experiment.

DISCUSSION

Capsule and biofilm formation in prokaryotes play important roles in human pathogenesis and both factors have been attributed to antibiotic resistance in persistent infections. The purpose of this study was to study the relationship between capsule formation and the formation of biofilm.

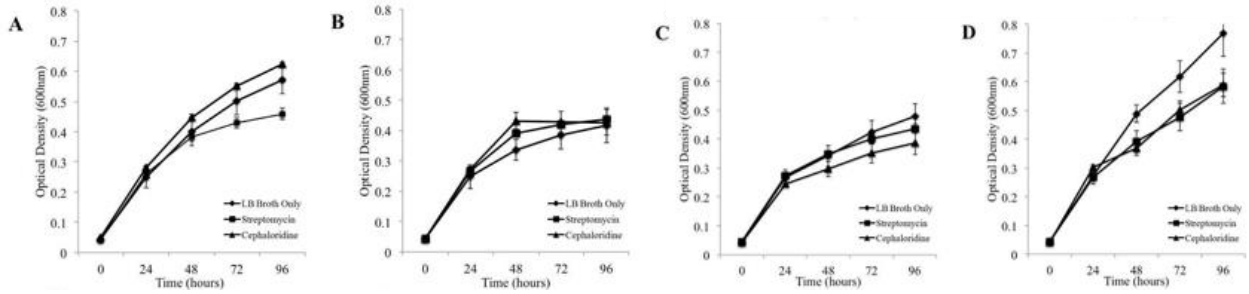

FIG 2 Growth curves measured at OD_{600} of the *E. coli* strains in a 96-well plate incubated at 21°C for 96 hours. (A) WT strain (B) Δ*wza* mutant (C) Δ*cpsB* mutant (D) CWG655 strain.

As seen in Figure 1, the amounts of capsular polysaccharides quantified under sub-MIC cephaloridine treatment are higher across all strains compared to the other treatments. Cephaloridine is a beta-lactam antibiotic, which acts by inhibiting the peptidoglycan synthesis pathway and ultimately causing lysis of the cell by osmotic pressures (18). These results agree with previous studies and indicate that *cps* operon expression is upregulated upon abnormal alterations to the bacterial cell wall (12).

A relationship between sub-MIC induced capsule and biofilm formation can be made by contrasting the same samples in Figures 1 and 3. For the *E. coli* K-12 WT samples, we observed that those grown in LB containing sub-MIC antibiotics had higher amounts of CPS compared to those grown in LB. A similar result was found with biofilm quantification, and WT strains treated with sub-MIC antibiotics had increased biofilm in contrast to WT grown in LB. Compared to *E. coli* K-12 WT, all the capsule mutant strains had lower amounts of total carbohydrates both in the presence and absence of sub-MIC of antibiotics. A corresponding reduction in biofilm formation can be seen in the Δ*wza* mutant and CWG655 mutant. These results indicate a positive correlation between capsule production and biofilm formation, which is not surprising as several polysaccharides, including colanic acid, contribute to the production of capsule and are involved in the biofilm phenotype by forming the overall composition of the biofilm matrix (19). Ultimately, these polysaccharides aid in the development of the biofilm by providing resistance to desiccation (7).

The Δ*cpsB* mutant produced less capsule compared to the *E.coli* WT strains when pretreated with sub-MIC antibiotics (Figure 1). The Δ*cpsB* mutant is deficient in colanic acid, a component of capsule and biofilms, and thus it is logical that a decreased amount of capsule is observed. However, Figure 3 demonstrates that all the conditions involving the Δ*cpsB* mutant produced greater amounts of biofilm compared to the Δ*wza* and CWG655 mutant samples. The deficiency in producing colanic acid did not impair the mutant's ability to produce

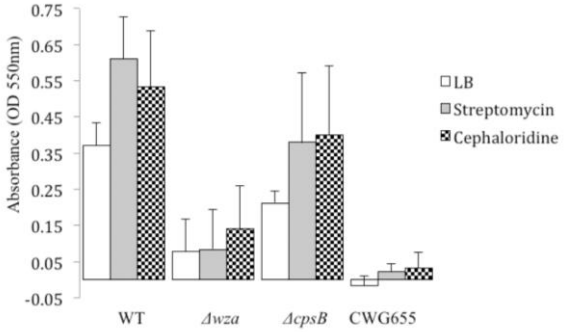

FIG 3 Biofilm formation in *E.coli* K-12 WT and Δ*wza*, Δ*cpsB*, CWG655 mutants induced by sub-MIC of streptomycin and cephaloridine at 0.5 µg/ml. The amount of biofilm was quantified using a crystal violet staining assay after 96 hours of incubation at 21°C. The biofilm-staining crystal violet dye was dissolved in 30% acetic acid and the absorbance of 10-time diluted acetic acid was measured. The 550nm reading of the LB control was subtracted from all samples when plotting. The error bars were calculated as the standard error of seven replicates.

biofilm as much as the other mutant types. A previous study has also demonstrated that Δ*cps* mutants are not deficient in biofilm production. The study showed that colanic acid is not required for biofilm development, but instead influences the composition of the biofilm (20). They concluded that Δ*cps* mutants had thinner biofilms with cells more tightly packed compared to wildtype (20). Thus, in our study, decreased levels of capsule in the Δ*cpsB* mutant compared to WT may be due to different biofilm architecture, and further research should be conducted to confirm these results.

The CWG655 mutant had sufficient total cell growth in LB media and LB media containing sub-MIC of streptomycin and cephaloridine after 96 hours of incubation (Figure 2). However, the CWG655 mutant was completely deficient in biofilm formation in both the presence and absence of sub-MIC of antibiotics (Figure 2). Since the CWG655 mutant had a complete knockout of the *wza-wzb-wzc* gene cluster, the lack of biofilm formation in this strain suggests that the Wzy-transporter

contributes to the ability of planktonic cells to attach to the surface and establish steady biofilm. Previous studies have demonstrated that Wza, Wzb, and Wzc proteins of the Wzy-system function coordinately in the assembly of capsule which increases adherence to host tissues (8). Partial deficiency in biofilm formation was observed in the Δ*wza* mutant, which has a deletion in one component of the Wzy-transporter. This suggests that the outer membrane channel formed by the Wza oligomeric lipoprotein was involved in biofilm formation and it might be worthwhile to study the role of the other components of the Wzy-transporter including *wzb* and *wzc*.

From our results, we can conclude that sub-MIC levels of antibiotics induces capsule production and biofilm formation where activation of the capsule biosynthesis pathway may also promote biofilm formation. The WT displayed greater CPS and biofilm levels under sub-MIC antibiotic conditions, compared to the untreated samples. Also, the amount of the biofilm produced appears to be more dependent on the Wzy-transporter than on the *cpsB* gene.

FUTURE DIRECTIONS

Future studies should focus on the genes knocked out in the CWG655 strain, as it shows decreased capsule levels, along with no detected biofilm formation. This would likely involve obtaining *E. coli* K-12 mutants with deletions in *wzb* or *wzc* and performing biofilm growth analysis to determine which of these genes would play a role in biofilm formation. Gene complementation analysis would confirm the results of the biofilm assay. A reverted WT phenotype by complementing Δ*wza*, Δ*wzb and* Δ*wzc* mutants with corresponding genes would allow concurrent studies to further narrow down the selection of genes that may be involved in both capsule and biofilm formation. Additionally, we have demonstrated that Δ*cpsB* mutants had intermediate levels of biofilm formation compared to that of the other experimental strains. Fluorescence microscopy could be used to compare biofilm architecture between the WT and Δ*cpsB* mutant. This would help to further examine the relationship colanic acid has with biofilm formation and how it influences biofilm architecture.

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REFERENCES

1. **Lindsay D, von Holy A.** 2006. Bacterial biofilms within the clinical setting: what healthcare professionals should know. J. Hosp. Infect. **64:**313-325.
2. **Reisner A, Haagensen JA, Schembri MA, Zechner EL, Molin S.** 2003. Development and maturation of *Escherichia coli* K-12 biofilms. Mol. Microbiol. **48:**933-946.
3. **Danese PN, Pratt LA, Kolter R.** 2000. Exopolysaccharide Production Is Required for Development of *Escherichia coli* K-12 Biofilm Architecture. J. of Bacteriol. **182:**3593-3596.
4. **Van Houdt R, Michiels CW.** 2005. Role of bacterial cell surface structures in *Escherichia coli* biofilm formation. Res. Microbiol. **156:**626-633.
5. **Stevenson G, Andrianopoulos K, Hobbs, M, Reeves, PR.** 1996. Organization of the *Escherichia coli* K-12 gene cluster responsible for production of the extracellular polysaccharide colanic acid. J. Bacteriol. **178:**4885-4893.
6. **Ganal S, Gaudin C, Roensch K, Tran M.** 2007. Effects of streptomycin and kanamycin on the production of capsular polysaccharides in *Escherichia coli* B23 cells. J. Exp. Microbiol. Immunol. **11:**54-59.
7. **Whitfield C.** 2006. Biosynthesis and assembly of capsular polysaccharides in *Escherichia coli*. Annu. Rev. Biochem. **75:**39-68.
8. **Reid AN, Whitfield C.** 2005. Functional analysis of conserved gene products involved in assembly of *Escherichia coli* capsules and exopolysaccharides: evidence for molecular recognition between Wza and Wzc for colanic acid biosynthesis. J. Bacteriol. **187:**5470-5481.
9. **Naimi I, Nazer M, Ong L, Thong E.** 2009. The role of *wza* in extracellular capsular polysaccharide levels during exposure to sublethal doses of streptomycin. J. Exp. Microbiol. Immunol. Vol. **13:**36-40.
10. **Ganal S, Gaudin C, Roensch K, Tran M.** 2007. Effects of streptomycin and kanamycin on the production of capsular polysaccharides in *Escherichia coli* B23 cells. J. Exp. Microbiol. Immunol. **11:**54-59.
11. **Hoffman LR, D'Argenio DA, MacCoss MJ, Zhang Z, Jones RA, Miller SI.** 2005. Aminoglycoside antibiotics induce bacterial biofilm formation. Nature. **436:**1171-1175.
12. **Sailer FC, Meberg BM, Young KD.** 2003. β-Lactam induction of colanic acid gene expression in *Escherichia coli*. FEMS Microbiol. Lett. **226:**245-249.
13. **Geisinger E, Isberg RR.** 2015. Antibiotic Modulation of Capsular Exopolysaccharide and Virulence in *Acinetobacter baumannii*. PLoS Pathogens. **11:**e1004691.
14. **Brimacombe CA, Beatty JT.** 2013. Surface Polysaccharide Extraction and Quantification. Bio-protocol. **3**(20)**:** e934. http://www.bio-protocol.org/e934
15. **Beishir L.** 1996. Microbiology in practice: a self-instructional laboratory course. Benjamin-Cummings Publishing Company.
16. **Ren D, Zuo R, Gonzalez-Barrios AF, Bedzyk LA, Eldridge GR, Pasmore ME, Wood TK.** 2005. Differential gene expression for investigation of *Escherichia coli* biofilm inhibition by plant extract ursolic acid. Appl. Environ. Microbiol. **71:**4022-4034.
17. **O'Toole GA.** 2011. Microtiter dish biofilm formation assay. J. Vis. Exp. **47**:2437.
18. **Mandell GL.** 1973. Cephaloridine. Ann. Intern. Med. **79:**561-565.
19. **Beloin C, Roux A, Ghigo J.** 2008. *Escherichia coli* biofilms. Curr. Top. Microbiol. Immunol. **322:**249-289.
20. **Prigent-Combaret C, Prensier G, Le Thi TT, Vidal O, Lejeune P, Dorel C.** 2000. Developmental pathway for biofilm formation in curli-producing *Escherichia coli* strains: role of flagella, curli and colanic acid. Environ. Microbiol. **2:**450-464.